Objection to the disclosure

The Examiner stated that the prior objection to the disclosure is maintained for the reasons as set forth in the last Office Action mailed June 19, 1998 (see Paper No. 16). The Examiner stated that applicants submit they will provide a new Figure 6B to overcome the rejection when the case is in condition for allowance. The Examiner stated that until applicants submit a proper Figure said objection is maintained.

In response, applicants will provide a new figure 6B upon the indication of allowable subject matter.

Obvious type double patenting rejection

The Examiner rejected claims 97-118 as being unpatentable over the all the claims of copending application No. 08/477,097. The Examiner stated that applicants' assert that the added new claims in the instant application and in the copending application obviate the obvious type double patenting. The Examiner stated this is not persuasive, the specific conjugated ganglioside derivatives (GM2 or GD2) herein anticipate the generic conjugates of GM2 and GD2 of claims 79-99 in the '097 application. The Examiner stated that applicants' amendments are insufficient to obviate the rejection.

The Examiner provisionally rejected claims 97-118 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 78-100 of copending application No. 08/475,784. The Examiner stated that applicants assert that the added new claims in the instant application and in the copending

application obviate the obvious type double patenting. The Examiner stated this is not persuasive, the specific conjugated ganglioside derivatives (GM2 or GD2) herein anticipate the conjugates of GM2 and GD2 of claims 78-100 in the '784 application. The Examiner stated additionally, the GM2 conjugate of the instant application render obvious the other ganglioside conjugates GM3, GD3, GD3 lactone, O-acetyl GD3 and GT3 of the '784 application in view of Livingston et al (U.S. Patent No. 5, 102,663) and teach that gangliosides GM3, GM2, GD3, GD2, GT3 and O-aceytl GD3 are ganglioside that are prominent cell-membrane components of melanoma and other tumors of neuroectodermal origin (column 1, lines 22-28) are useful to initiate an immune response. The Examiner stated applicants' amendments are insufficient to obviate the rejection.

In response, applicants respectfully traverse the Examiner's above rejection. Applicants contend that the claims of the cited applications do not render obvious the claims of the subject application and therefore, an obviousness-type double patenting rejection is not appropriate. Applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Rejection under 35 U.S.C. 112, first paragraph

The Examiner rejected claims 97-99 and 101-118 under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention for the reasons set forth in the Office Action mailed 10/06/99 (see Paper No. 20)

for claims 69-71 and 72-96.

The Examiner stated applicants' arguments' and amendments have been carefully considered. The Examiner stated the claims still recite "derivatives of KLH". The Examiner stated such derivatives are not enabled for reasons already made of record. The Examiner stated applicants' arguments and amendments are insufficient to obviate this rejection.

The Examiner stated as to new claims 112-118, the claims are enabled for the use of the composition only for the treatment of cancer but are not enabled for the prevention of cancer, for reasons made of record in Paper No. 8, mailed 6-13-96. The Examiner stated applicants' argue that the conjugate vaccine of the invention prevents outgrowth of micrometastases and prevents cancer *per se* (Zhang et al, Cancer Research 58:2844-2849, 1998). The Examiner stated this is not persuasive, the claims are not drawn to preventing outgrowth of micrometastases and the conjugate used in the paper is GD2-KLH (10 ug of GD2 conjugated to 60 ug KLH, wherein the conjugation of GD2 of KLH was achieved by conversion of the GD2 ceramide double bond to aldehyde by ozonolysis and attachment to KLH by reductive amination in the presence of cyanoborohydride) plus 10 ug QS-21. The Examiner stated thus, the conjugate of the claims is not that which has been demonstrated by the art prevents outgrowth of micrometastases, nor does the method provide for the method of the paper (multiple doses administered by a specific route. The Examiner stated moreover, the article specifically teach that the vaccine"...should be used exclusively in the adjuvant setting, where circulating tumor ells and micrometastases

are the primary targets (page 2844, last line of abstract)." The Examiner stated the evidence of the paper targeted circulating cells specific type of tumor cell (lymphoma) which was administered intravenously and micrometastases thereof from circulation, which is clearly not representative of cancers or relapses as instantly claimed. The Examiner stated moreover, figure 1, demonstrates that administration of the GD2-KLH, QS-21 vaccine at days -21, -14 and -7 does not prevent cancer as demonstrated by the death of some of the experimental group after experimental intravenous challenge of lymphoma cells (see Figure 1, Experiments 3 and 6B). The Examiner stated at page 2845, column 2, second and third paragraph, Zhang et al teach that the vaccine prolonged survival, but in the discussion of experiment 6, only 4 out of 6 vaccinated mice remained disease free at the latest time point measured. The Examiner stated moreover, Zhang et al admit that the alleged protection in Experiment 7 of Figure 1, was "not statistically significant" and moreover this experiment is not directly comparable with the other experiments because the tumor burden administered intravenously was substantially reduced. The Examiner stated clearly the vaccine when administered prior to the cancer does not prevent as claimed or as argued by applicants. The Examiner stated additionally, prevention of relapse as claimed has not been demonstrated nor specifically addressed by this paper and Zhang et al admits that "If antibodies of sufficient titer and potency to eliminate circulating cancer cells and micrometastases could be maintained in cancer patients as well, even metastatic cancer would have quite a different implication. The Examiner stated with continuing showers of metastases no longer possible, aggressive treatment of primary and metastatic sites might result in long term control." The

Examiner stated relapsing of cancer is quite different than elimination of micrometastases and the paper only addresses circulating syngeneic tumor lymphoma cells and micrometastases (see page 2848, column 1, last paragraph) not primary cancer. The Examiner state Zhang et al do not address primary cancer and the experimental protocols set forth therein do not address prevention of primary cancer as is claimed for prevention of relapse of cancer. The Examiner stated reduction of circulating lymphoma cells and reduction in micrometastases is not commensurate in scope with prevention of cancer or prevention of a relapse of cancer.

In response, with respect to the Examiner's above rejection concerning KLH derivatives, applicants respectfully traverse the Examiner's rejection. Claims which recite "derivatives of KLH" are enabled. Applicants respectfully direct the Examiner's attention to page 12, lines 4-13 for examples of generating such derivatives. For example, one skilled in the art could generate a KLH derivative by directly liking it to an immunological adjuvant, such as monophospholipid A, non-ionic block copolymers or a cytokine, as taught by on page 12, lines 4-13 of the specification. Nevertheless, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove amended the claims such that they no longer recite the term "derivative thereof" with respect to KLH.

With respect to the Examiner's above rejection concerning the prevention of cancer, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove canceled claim 112 without

disclaimer or prejudice to their right to pursue the subject matter of this claim in a later-filed application. In addition, applicants acknowledge the Examiner's statement that the claims are enabled for the use of the composition for treatment. Accordingly, applicants have also hereinabove amended claim 113 such that it now recites a "method of treating a cancer in a subject." Therefore, the claimed invention is enabled.

Applicants contend that these amendments obviate the above rejection and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Rejection under 35 U.S.C. 103(a)

The Examiner rejected claims 97-113 and 115-118 under 35 U.S.C. 103(a) as being unpatentable over Livingston et al. (Cancer Research, 149:7045-7050, 1989) in view of Ritter et al. (Seminars in Cancer Biology, 2:401-409, 1991), Liane et al (Journal of Biological Chemistry, 249(14):4460-4466, 1974), Livingston et al. (U.S. Patent No. 5,102,663), Ritter et al. (Immunobiol, 182:32-43, 1990), Kensil et al. (The Journal of Immunology, 146(2):431-437, 1991), and Marciani et al. (Vaccine, 9:89-96, 1991) and Uemura et al (J Biochem, 79(6):1253-1261, 1976) for reasons made of record for previous claims 69-81 and 83-96 in Paper No. 20, 10-6-99. The Examiner stated applicants' arguments have been carefully considered but are not persuasive. The Examiner stated applicants' contend that the references neither alone nor in combination teach the claimed invention of conjugation of the ganglioside derivative through a ceramide derived carbon. The Examiner stated this is not persuasive, the conjugation procedure as combined provides a

reasonable expectation of success as demonstrated by Uemura et al which demonstrates the ozonolysis and reduction of various sphingolipids did not affect the haptenic reactivity with antibodies. The Examiner stated applicants' have neither pointed distinguishing features of applicants invention nor provided any scientific evidence or rationale which would indicate that the conjugation procedure as combined by the prior art would not arrived at the claimed product and methods. The Examiner stated applicants arguments are not persuasive and the rejection stands across the new claims.

In response, applicants respectfully traverse the Examiner's above rejection. Applicants respectfully disagree with the Examiner's contention that the conjugation procedure as combined provides for the identical procedure as applicants' coupling procedure. Applicants contend that the cited references, namely Livingston et al. (Cancer Research) in view of Ritter et al. (Seminars in Cancer Biology), Liane et al (Journal of Biological Chemistry), Livingston et al. (U.S. Patent No. 5,102,663), Ritter et al. (Immunobiol), Kensil et al. (The Journal of Immunology), and Marciani et al. (Vaccine) and Uemura et al (J Biochem) does not teach, suggest or disclose applicants claimed invention and therefore do not render obvious the claimed invention.

Applicants point out that newly amended claim 97 recites a composition which comprises: a) conjugate of i) a GM2 or GD2 ganglioside derivative which comprises an unaltered oligosaccharide part and an altered ceramide portion comprising a sphingosine base, to ii) Keyhole Limpet Hemocyanin comprising an ε-aminolysyl group;

b) a saponin derivable from the bark of a Quillaja saponaria Molina tree; and c) a pharmaceutically acceptable carrier; the relative amounts of such conjugate and such saponin being effective to stimulate or enhance antibody production in a subject, **wherein in the conjugate the ganglioside derivative is conjugated to Keyhole Limpet Hemocyanin through a C-4 carbon of the sphingosine base of the ceramide portion of the ganglioside derivative to the ε-aminolysyl group of Keyhole Limpet Hemocyanin** [emphasis added].

First, the Examiner acknowledges that the primary reference, i.e. Livingston et al. Cancer Research 1989, ("Livingston 1989") does not teach conjugation of GM2 or other gangliosides by means of a carbon on the ceramide moiety with aminolysyl groups on KLH in a composition or using this method for treatment (see October 6, 1999 Office Action, page 8).

To compensate for the lack of such disclosure, the Examiner relies relies primarily on two references, namely Ritter et al., Cancer Biology 1991 ("Ritter 1991") and Ritter et al., Immunobiology 1990 ("Ritter 1990"). However, applicants submit that neither of these references supplies what it missing from the primary reference.

Ritter 1991 discloses on page 406, column 1 two approaches for augmenting the immunogenicity of gangliosides in a mouse, and states that only one of these approaches is capable of inducing consistent IgG antibodies to gangliosides in the mouse. Ritter 1991 describes this approach as covalently attaching gangliosides to foreign carrier proteins such as KLH.

Although Ritter 1991 refers to the conjugation of GM2 to KLH, there is no description of the chemical nature of the conjugate or of how to make the conjugate. Thus, Ritter 1991 neither discloses anything conjugated through the ceramide, nor enables making any such conjugate. Applicants respectfully direct the Examiner's attention to the highlighted portion of claim 97 above relating to the conjugation, which recites "...wherein in the conjugate the ganglioside derivative is conjugated to Keyhole Limpet Hemocyanin through a C-4 carbon of the sphingosine base of the ceramide portion of the ganglioside derivative to the ε-aminolysyl group of Keyhole Limpet Hemocyanin..." The Examiner's indication that one skilled in the art would interpret Ritter 1991 to involve ceramide conjugation is only speculation. Based on Ritter 1991, one skiiled in the art would not understand that the linkage would be through the ceramide. The Examiner tries to justify that the references teach linkages through the ceramide by using Ritter 1990. However, Ritter 1990 does **not** teach conjugation in a ceramide region.

Ritter 1990 describes making chemical derivatives of GD3. The four derivatives described in Table 1 on page 34 are as follows: (1)amide, which is not immunoreactive with monoclonal antibodies to native GD3; (2) gangliosidol, which is not immunoreactive with monoclonal antibodies to native GD3; (3) lactone I, which is reactive but less than native GD3; and (4)lactone II, which is also reactive but less than native GD3. In Ritter 1990, there is no discussion of a conjugation to KLH. There is merely a description of chemical modifications of the ganglioside. Applicants point out to the Examiner that these derivatives are in the carbohydrate portion and not in the ceramide.

Based on the Table 4 in Ritter 1990, one would interpret that for GD3, the preference is to make a Lactone 1 derivative, which is a lactone chemically derivatized in the carbohydrate since it is more immunogenic than GD3 itself. Based on Ritter 1990, one would probably make a Lactone derivative. However, there is no suggestion of using the ceramide for such derivative.

The Examiner's attempt to use Ritter 1990 to support his obviousness speculation is incorrect because: (1) Ritter 1990 discloses that the conjugation is through the carbohydrate, not the ceramide; and (2) Ritter 1990 teaches away from ceramide conjugation and indicates that conjugation through a lactone is preferred.

Thus, there is neither a specific disclosure, nor is it obvious from either Ritter 1990 or Ritter 1991 to conjugate through the ceramide. Accordingly, the primary reference (i.e. Livingston et al.) in view of Ritter 1990 and Ritter 1991 does not teach, suggest or disclose the claimed invention Moreover, the other cited references do not supply what is missing from either the primary reference, Ritter 1990 or Ritter 1991.

The Examiner cited Uemura et al as disclosing that ozonolysis and reduction of various sphingolipids do not affect the haptenic activity with antibodies. The Examiner stated that the combination [of references] provides a reasonable expectation of success as demonstrated by Uemura et al which demonstrates the ozonolysis and reduction of various sphingolipids did not affect the haptenic reactivity with antibodies. However, Uemura et al does not supply

what is missing from the primary reference with respect to conjugation through a ceramide-derived carbon to a carrier protein.

The Examiner cited Kensil and Marciani for their disclosures with respect to QS-21. The Examiner cited Livingston et al. (U.S. Patent No. 5,102,663) with respect to various gangliosides being cell membrane components of melanoma. Accordingly, neither of these references disclose what is missing from the primary reference with respect to conjugation through a ceramide-derived carbon to a carrier protein.

The Examiner cited Liane et al (Journal of Biological Chemistry), alleging that it "teaches a method for covalent coupling of gangliosides to amino ethyl agarose or the amino group bearing glass beads by oxidative ozonolysis of the olefinic bond of the spingosine moiety (i.e. the instant carbon double bond of ceramide) and coupling of the carboxyl bearing product to the amino group bearing glass beads." Applicants submit that Liane does not supply what is missing from the primary reference with respect to conjugation through a ceramide-derived carbon. In support, applicants attach hereto as **Exhibit B** a copy of Helling et al., Cancer Research 54: 197-203, which was cited as reference 3 in an information disclosure statement filed on May 2, 1997 in connection with the subject application. Applicants point out that Helling et al. addresses the cited reference (i.e. Liane et al.) on page 201, second paragraph of the discussion stating the "earlier" Liane et al. method

> is of limited use for the conjugation of gangliosides to carrier proteins because it requires acetylated, methyl ester derivatives of gangliosides to avoid coupling via

> the sialic acid carboxyl group. Deacylation after conjugation under basic conditions is necessary, conditions most proteins cannot be exposed to without degradation.

Applicants point out that the claimed invention recites in part "...a conjugate of i) a GM2 or GD2 ganglioside derivative which comprises an unaltered oligosaccharide part and an altered ceramide portion comprising a sphingosine base, to ii) Keyhole Limpet Hemocyanin..." Keyhole Limpet Hemocyanin is a carrier protein and accordingly, the Liane et al. methods would not enable a conjugation such as that recited in the claims because the Liane et al. conditions would result in protein degradation. Accordingly Liane et al. does not provide what is missing from the primary reference, i.e. a teaching of a conjugation of a ganglioside to a protein through a ceramide derived carbon, and an enabling disclosure of how to do so. Therefore, the primary reference in view of Liane et al. does not teach, suggest or disclose the claimed invention.

Accordingly, the primary reference, i.e. Livingston 1989 in view of the other cited references, namely Ritter 1990, Liane et al (Journal of Biological Chemistry), Livingston et al. (U.S. Patent No. 5,102,663), Ritter 1991, Kensil et al. (The Journal of Immunology), and Marciani et al. (Vaccine) and Uemura et al (J Biochem) does not render obvious the applicants' claimed invention. Applicants contend that these remarks obviate the above rejection and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Rejection under 35 U.S.C. 103(a)

The Examiner rejected claim 114 under 35 U.S.C. 103(a) as being unpatentable over Livingston et al. (Cancer Research), Ritter et al. (Cancer Biology, 1991), Liane et al (Journal of Biological Chemistry), Livingston et al. (U.S. Patent No. 5,102,663), Ritter et al. (1990), Kensil et al, and Marciani et al., and Uemura et al (J Biochem) as applied to claims 69-81 and 83-96 above and further in view of Irie et al. (U.S. Patent Nol 4,557,931) for reasons made of record for claim 82 in Paper No. 20, mailed 10-6-99. The Examiner stated that applicants' arguments have been carefully considered but are not persuasive. The Examiner stated that applicants' contend that the references neither alone nor in combination teach the claimed invention of conjugation of the ganglioside derivative through a ceramide derived carbon. The Examiner stated this is not persuasive, the conjugation procedure as combined provides for the identical procedures as Applicants' coupling procedure. Moreover, the combination provides a reasonable expectation of success as demonstrated by Uemura et al which demonstrates the ozonolysis and reduction of various sphingolipids did not affect the haptenic reactivity with antibodies. The Examiner stated applicants' have neither pointed distinguishing features of applicants invention nor provided any scientific evidence or rationale which would indicate that the conjugation procedure as combined by the prior art would not arrived at the claimed product and methods. The Examiner stated Applicants arguments are not persuasive and the rejection stands across the new claims.

In response, applicants respectfully traverse the Examiner's above

rejection for the reasons stated above supra on pages 11-16. Applicants contend that Irie does not supply what is missing from either the primary reference or any of the other references, i.e. with respect to conjugation of gangliosides through a ceramide-derived carbon to carrier proteins. Accordingly Livingston et al. (Cancer Research), Ritter et al. (Cancer Biology, 1991), Liane et al (Journal of Biological Chemistry), Livingston et al. (U.S. Patent No. 5,102,663), Ritter et al. (1990), Kensil et al, and Marciani et al., and Uemura et al (J Biochem) as applied to claims 69-81 and 83-96 above and further in view of Irie et al. (U.S. Patent No. 4,557,931) does not teach, suggest or disclose applicants' claimed invention and therefore do not render obvious the claimed invention. Applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Summary

For the reasons set forth hereinabove, applicants respectfully request that the Examiner reconsider and withdraw the various grounds of objection and rejection and earnestly solicit allowance of the now pending claims, i.e. claims 97-99, 101-111 and 113-118.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone at the number provided below.

No fee is deemed necessary in connection with the filing of this Amendment. However, if any fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,

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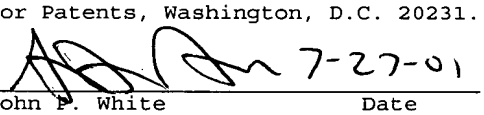
I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.



7-27-01

John P. White — Date
Reg. No. 28,678
Spencer H. Schneider
Reg. No. 45,923

**Exhibit A**

--97. (Amended) A composition which comprises:

a) a conjugate of i) a GM2 or GD2 ganglioside derivative which comprises an unaltered oligosaccharide part and an altered ceramide portion comprising a sphingosine base, to ii) Keyhole Limpet Hemocyanin [or a derivative thereof] comprising an ε-aminolysyl group;

b) a saponin derivable from the bark of a Quillaja saponaria Molina tree; and

c) a pharmaceutically acceptable carrier;

the relative amounts of such conjugate and such saponin being effective to stimulate or enhance antibody production in a subject,

wherein in the conjugate the ganglioside derivative is conjugated to Keyhole Limpet Hemocyanin [or the derivative thereof] through a C-4 carbon of the sphingosine base of the ceramide portion of the ganglioside derivative to the ε-aminolysyl group of Keyhole Limpet Hemocyanin [or the derivative thereof].--

--110. (Amended) The composition of claim 97, wherein the molar ratio of the ganglioside derivative to Keyhole Limpet Hemocyanin [or the derivative thereof] is between about 200 and about 1400.--

--111. (Amended) A method of stimulating or enhancing antibody production in a subject which comprises administering to the subject an effective amount of a composition which

comprises:

a) a conjugate of i) a GM2 or GD2 ganglioside derivative which comprises an unaltered oligosaccharide part and an altered ceramide portion comprising a sphingosine base, to ii) Keyhole Limpet Hemocyanin [or a derivative thereof] comprising an ε-aminolysyl group;

b) a saponin derivable from the bark of a Quillaja saponaria Molina tree; and

c) a pharmaceutically acceptable carrier;

the relative amounts of such conjugate and such saponin being effective to stimulate or enhance antibody production in the subject,

wherein in the conjugate the ganglioside derivative is conjugated to Keyhole Limpet Hemocyanin [or the derivative thereof] through a C-4 carbon of the sphingosine base of the ceramide portion of the ganglioside derivative to the ε-aminolysyl group of Keyhole Limpet Hemocyanin [or the derivative thereof], so as to thereby stimulate or enhance antibody production in the subject.--

--113. (Amended) A method of [preventing or] treating a cancer in a subject which comprises administering to the subject an effective cancer [preventing or] treating amount of a composition which comprises:

a) a conjugate of i) a GM2 or GD2 ganglioside derivative which comprises an unaltered oligosaccharide part and an altered ceramide portion comprising a sphingosine base, to ii)

Keyhole Limpet Hemocyanin [or a derivative thereof] comprising an ε-aminolysyl group;

b) a saponin derivable from the bark of a Quillaja saponaria Molina tree; and

c) a pharmaceutically acceptable carrier;

the relative amounts of such conjugate and such saponin being effective to stimulate or enhance antibody production in the subject,

wherein in the conjugate the ganglioside derivative is conjugated to Keyhole Limpet Hemocyanin [or the derivative thereof] through a C-4 carbon of the sphingosine base of the ceramide portion of the ganglioside derivative to the ε-aminolysyl group of Keyhole Limpet Hemocyanin [or the derivative thereof], so as to thereby [prevent or] treat [a] <u>the</u> cancer in the subject.--

--114. (Amended) The method of claim [112 or] 113, wherein the cancer is of epithelial origin.--

--115. (Amended) The method of claim [112 or] 113, wherein the cancer is of neuroectodermal origin.--

--117. (Amended) The method of [any one of claims 111-] <u>claim 111 or</u> 113, wherein the administering is effected at two or more sites.--

[CANCER RESEARCH 54, 197-203, January

# $G_{D3}$ Vaccines for Melanoma: Superior Immunogenicity of Keyhole Limpet Hemocyanin Conjugate Vaccines[1]

**Friedhelm Helling,[2] Ann Shang, Michele Calves, Shengle Zhang, Shunlin Ren, Robert K. Yu, Herbert F. Oettgen, and Philip O. Livingston**

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## ABSTRACT

**Cell surface gangliosides show altered patterns of expression as a consequence of malignant transformation and have therefore been of interest as potential targets for immunotherapy, including vaccine construction. One obstacle has been that some of the gangliosides that are overexpressed in human cancers are poorly immunogenic in humans. A case in point is $G_{D3}$, a prominent ganglioside of human malignant melanoma. Using an approach that has been effective in the construction of bacterial carbohydrate vaccines, we have succeeded in increasing the immunogenicity of $G_{D3}$ in the mouse by conjugating the ganglioside with immunogenic carriers. Several conjugation methods were used. The optimal procedure involved ozone cleavage of the double bond of $G_{D3}$ in the ceramide backbone, introducing an aldehyde group, and coupling to aminolysyl groups of proteins by reductive amination. Conjugates were constructed with a synthetic multiple antigenic peptide expressing repeats of a malarial T-cell epitope, outer membrane proteins of *Neisseria meningitidis*, cationized bovine serum albumin, keyhole limpet hemocyanin, and polylysine. Mice immunized with these conjugates showed a stronger antibody response to $G_{D3}$ than mice immunized with unconjugated $G_{D3}$. The strongest response was observed in mice immunized with the keyhole limpet hemocyanin conjugate of the $G_{D3}$ aldehyde derivative and the adjuvant QS-21. These mice showed not only a long-lasting high-titer IgM response but also a consistent high-titer IgG response (predominantly IgG1), indicating recruitment of T-cell help, although the titers of IgM and IgG antibodies following booster immunizations were not as high as they are in the response to classical T-cell-dependent antigens. This method is applicable to other gangliosides, and it may be useful in the construction of immunogenic ganglioside vaccines for the immunotherapy of human cancers expressing gangliosides on their cell surface.**

## INTRODUCTION

Gangliosides are glycolipid constituents of the cell membrane. The term was coined in 1942 to refer to lipids of the central nervous system that contained sialic acid, to signify their prime location in ganglion cells and their glycosidic nature (1). Their lipophilic component, the ceramide (an amide-linked long-chain sphingoid base and a fatty acid), is thought to be embedded in the outer membrane of the cell membrane lipid bilayer. The carbohydrate portion of the molecule is oriented toward the outside of the cell. Malignant transformation appears to activate enzymes involved in ganglioside glycosylation, resulting in altered patterns of ganglioside expression in tumors such as astrocytoma, neuroblastoma, and malignant melanoma (2). In normal melanocytes, for example, the predominant ganglioside is $G_{M3}$.[3] Other gangliosides including $G_{D3}$, $G_{M2}$, $G_{D1a}$, and $G_{T1b}$ constitute less than 10% of the total (3). In malignant melanoma, increased expression of $G_{D3}$, $G_{D2}$, and $G_{M2}$ has been observed (4, 5), and these gangliosides have therefore been considered potential targets for immunotherapy.

One approach to ganglioside-targeted immunotherapy has been the use of mAbs.[4] Treatment of patients with melanoma or neuroblastoma with mAb recognizing $G_{D3}$, $G_{D2}$, or $G_{M2}$ has resulted in tumor regression in some cases (6–9). The other approach has been to immunize patients with ganglioside vaccines in attempts to induce production of ganglioside antibodies by the patients themselves. These attempts have been successful so far only with $G_{M2}$ vaccines. Patients with American Joint Committee on Cancer Stage III malignant melanoma, after complete resection of all tumor, have been shown to produce anti-$G_{M2}$ antibodies in response to vaccination with $G_{M2}$ and *Bacillus Calmette-Guérin* (after pretreatment with low-dose cyclophosphamide to reduce suppressor activity), and the disease-free interval and overall survival were longer in patients producing $G_{M2}$ antibodies (10). $G_{D3}$ and $G_{D2}$, on the other hand, were found to be only rarely immunogenic when administered in the same way to patients with melanoma (11). Even with the $G_{M2}$ vaccines, the antibody response showed the characteristics of a T-cell-independent response, that is to say, IgM production of short duration, rare conversion to IgG production, and lack of a booster effect (12, 13).

Similar difficulties have been encountered in the development of effective vaccines against bacterial carbohydrate antigens. One approach that has been successful in overcoming these problems is conjugation of the antigen with immunogenic protein carriers. For example, a conjugate vaccine that links the *Haemophilus influenzae* type b capsular polysaccharide to the outer-membrane protein complex of *Neisseria meningitidis* serogroup B was recently shown to induce the production of antibodies and a high rate of protection against invasive disease caused by *Haemophilus influenzae* type b in infants (14), and similar results were reported for a conjugate vaccine using a nontoxic mutant diphtheria toxin as carrier (15).

We have explored this approach in attempts to increase the immunogenicity of melanoma gangliosides. We report here the effects of conjugating $G_{D3}$ with several protein carriers on its immunogenicity in the mouse.

## MATERIALS AND METHODS

**Gangliosides.** $G_{M3}$, $G_{M2}$ and $G_{D1b}$, extracted from bovine brain, were provided by Fidia Research Laboratory (Abano Terme, Italy). $G_{D2}$ was made from $G_{D1b}$ by enzymatic cleavage with β-galactosidase from bovine testes (16). $G_{D3}$ (mel) was isolated from human melanoma tissue (17), $G_{D3}$ (bbm) and GT3 were isolated from bovine buttermilk (18), and disialyllactose ($G_{D3}$ oligosaccharide) was isolated from bovine colostrum as previously described (19).

**Reagents.** HPTLC silica gel plates were obtained from E. Merck (Darmstadt, Germany); 4-chloro-1-naphthol, *p*-nitrophenyl phosphate disodium, and sodium cyanoborohydride were from Sigma Chemical Co. (St. Louis, MO);

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The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] To whom requests for reprints should be addressed, at Immunology Program, Memorial Sloan-Kettering Cancer Center, 425 East 68th Street, Room 820 K, New York, NY 10021.

[3] The designations $G_{M3}$, $G_{M2}$, $G_{M1}$, $G_{D3}$, $G_{D1a}$ and $G_{D1b}$ are used in accordance with the abreviated ganglioside nomenclature proposed by Svennerholm (40).

[4] The abbreviations used are: mAb, monoclonal antibody; MAP, multiple antigenic peptide; OMP, outer membrane protein; cBSA, cationized bovine serum albumin; ITLC, immune thin-layer chromatography; HPTLC, high-performance thin-layer chromatography; ELISA, enzyme-linked immunosorbent assays; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; bbm, bovine buttermilk.

methylsulfide was from Aldrich (Milwaukee, WI); cyclophosphamide (Cytoxan) was from Mead Johnson (Syracuse, NY); and QS-21 adjuvant, a homogeneous saponin component purified from *Quillaja saponaria* Molina tree (20), was kindly donated by Cambridge Biotech Corp (Worcester, MA). It is an amphipathic molecule and was provided as a white powder, forming a clear colorless solution when dissolved in PBS.

**Proteins.** Poly-L-lysine hydrobromide [MW(vis)3800] was purchased from Sigma, keyhole limpet hemocyanin (KLH) was from Calbiochem (La Jolla, CA), the cBSA-Imject Supercarrier immune modulator was from Pierce (Rockfort, IL), and *Neisseria meningitidis* OMPs were kindly provided by Dr. M. S. Blake (Rockefeller University, New York). MAP YAL-IV 294-I containing four repeats of a malarial T-cell epitope was a gift from Dr. J. P. Tam (Rockefeller University).

**Monoclonal Antibodies.** Rabbit anti-mouse immunoglobulins conjugated to horseradish peroxidase for ITLC, and rabbit anti-mouse IgM and IgG conjugated to alkaline phosphatase for ELISAs, were obtained from Zymed (San Francisco, CA); anti-$G_{D3}$ mAb R24 was generated in our laboratory (21).

**Serological Assays.** ELISA were performed as previously described (13). To control for nonspecific "stickiness," immune sera were also tested on plates to which no ganglioside had been added, and the reading was subtracted from the value obtained in the presence of ganglioside. The titer was defined as the highest dilution yielding a corrected absorbance of 0.1 or greater. Immunostaining of gangliosides with mAb or mouse sera was performed after separation on HPTLC silica gel glass plates as previously described (4). Plates were developed in solvent 1 [chloroform:methanol:water (0.25% $CaCl_2$), 50:40:10 (v/v)] or solvent 2 [ethanol:*n*-butylalcohol:pyridine:water:acetic acid, 100:10:10:30:3 (v/v)], and gangliosides were visualized with resorcinol-HCl reagent. Dot-blot immune stains were performed on nitrocellulose strips utilizing purified gangliosides spotted in equal amounts and developed as described before (13).

**Immunization.** Six-week-old female BALB/c × C57BL/6 $F_1$ mice (The Jackson Laboratory, Bar Harbor, ME) were given an i.p. injection of cyclophosphamide (15 mg/kg) 3 days before the first immunization and were then assigned to treatment groups. Groups of 4 or 5 mice were given three s.c. injections of a vaccine 2 weeks apart if not otherwise indicated. Each vaccine contained 20 μg $G_{D3}$ or 15 μg disialyllactose and 10 μg QS-21 in a total volume of 0.1 ml PBS. Mice were bled from the retroorbital sinus before vaccination and 2 weeks after the last vaccine injection unless indicated otherwise.

**$G_{D3}$ Conjugate Preparation.** $G_{D3}$ (2 mg) was dissolved in 2 ml methanol by sonication and cooled to −78°C in an ethanol/dry ice bath. Ozone was generated in an ozone generator (Del Industries, San Luis Obispo, CA) and was passed through the sample for 30 min under vigorous stirring (22, 23). The excess of ozone was then displaced with nitrogen over a period of 10 min. Methylsulfide (100 μl) was added (24), and the sample was kept at −78°C for 30 min and then at room temperature for 90 min under vigorous stirring. The sample was dried under a stream of nitrogen and monitored by HPTLC. The long-chain aldehyde was separated by adding *n*-hexane (2 ml) to the dry sample, followed by sonication for 5 min and centrifugation at 2000 × *g* for 15 min. The *n*-hexane was carefully drawn off and discarded, and the sample was dried under a stream of nitrogen. Cleaved $G_{D3}$ and native $G_{D3}$ were separated by HPLC (Waters, System 501, Milford, MA) utilizing a $C_{18}$ reversed-phase column (10 x 250 mm; Rainin Instruments, Ridgefield, NJ). Gangliosides were eluted with a linear water-acetonitrile gradient and monitored at 214 nm, and the fractions were analyzed by HPTLC. Fractions that contained cleaved $G_{D3}$ were combined and evaporated at 37°C with a Rotavapor (Büchi, Flawils, Switzerland). Cleaved $G_{D3}$ (1.5 mg), 1.5 mg protein carrier in PBS, and 2 mg sodium cyanoborohydride were incubated under gentle agitation at 37°C for 48 h. After 16 h 1 mg sodium cyanoborohydride was added. The progress of coupling was monitored by HPTLC. $G_{D3}$-protein conjugates did not migrate in solvent 1 and solvent 2 but remained at the origin as a resorcinol-positive band. The mixture was dialyzed across 5000 molecular weight cutoff dialysis tubing with three changes of PBS (4 liters each), at 4°C for 48 h, and passed through an Extractigel detergent-removing gel (Pierce, Rockfort, IL) for final purification of unconjugated $G_{D3}$. The samples were lyophilized, and their protein and ganglioside content was determined by BioRad protein assay and by neuraminic acid determination according to the method of Svennerholm (25).

**Disialyllactose Conjugate Preparation.** Disialyllactose was isolated from bovine colostrum as described previously (19). The carbohydrate was attached to protein by reductive amination (26). Disialyllactose (10 mg) was incubated with 2 mg proteins in 2 ml PBS for 14 days at 37°C after sterile filtration. Sodium cyanoborohydride (2 mg) was added at the beginning, and 1 mg was added every 3 days. The coupling was monitored by HPTLC in solvent 2. The disialyllactose conjugates were purified by dialysis across 5000 molecular weight cutoff dialysis membrane with three changes of PBS (4 liters each) at 4°C for 48 h, followed by lyophilization. The protein and neuraminic acid content was determined as described above. Disialyllactose was also conjugated to proteins according to the method described by Roy and Laferrière (27). During this procedure *N*-acroloylated glycopyranosylamine derivatives of the oligosaccharide were formed first, followed by conjugation via Michael addition to amino groups of the protein. Purification and determination of protein and neuraminic acid content were performed as described above.

**Determination of Antibody Subclasses.** Determination of antibody subclasses was performed by ELISA using subclass-specific rabbit anti-mouse immunoglobulins IgG1, IgG2a, IgG2b, IgG3, and IgA (Zymed, San Francisco, CA). Alkaline phosphatase-labeled goat anti-rabbit IgG served as the signal-generating reagent.

**FACS Analysis of Mouse Antisera.** A single cell suspension of the melanoma cell line SK-MEL-28 was obtained after treatment with 0.1% EDTA in PBS followed by passage through a 26½-gauge needle. Cells ($3 \times 10^5$) were incubated with 40 μl of 1:20 diluted post- or preimmunization serum for 30 min on ice. The cells were washed three times with 3% fetal calf serum in PBS. Thirty μl of diluted (1:50) fluorescein isothiocyanate-labeled goat anti-mouse IgG (Southern Biotechnology Associates Inc., Birmingham, AL) were added as secondary antibody, followed by incubation on ice for 30 min. Cells were washed three times as above and resuspended in 500 μl 3% fetal calf serum in PBS and analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA).

## RESULTS

**Preparation and Characterization of $G_{D3}$-Protein Conjugates.** $G_{D3}$ (bbm) in methanol was selectively cleaved with ozone at the C4-C5 double bond in the ceramide portion. It is assumed that methoxyperoxides are formed as intermediate products (24), and therefore methylsulfide was added as a reducing agent. The result of the cleavage was a $G_{D3}$ derivative with an aldehyde functional group in the position of the former double bond in the ceramide portion (Fig. 1). Cleaved $G_{D3}$ migrated slower than native $G_{D3}$, and formed double bands because the ceramide contained unsaturated fatty acids that were cleaved simultaneously (see Fig. 1, *inset*). Densitometric analysis of HPTLC plates showed that more than 70% of $G_{D3}$ (bbm) was cleaved by this procedure. Preliminary experiments involving longer ozone treatment had similar results, indicating that 30% of $G_{D3}$ from this source consists of sphinganine or phytosphingosine analogues that contain no ozone-cleavable ceramide double bond. Cleavage at −78°C with ozone treatment up to 1 h (depending on the amount of $G_{D3}$ used) was found to be optimal. Cleaved $G_{D3}$ persisted only in acidic and neutral phosphate buffers for up to 72 h, but with the formation of increasing amounts of oligosaccharide due to β-elimination reactions [which have been shown to occur much faster at alkaline pH (23)]. The decreased hydrophobicity of cleaved $G_{D3}$ compared to native $G_{D3}$ allowed its separation by HPLC on $C_{18}$ reversed-phase columns. Utilizing isocratic elution with a linear water-acetonitrile gradient, cleaved $G_{D3}$ was recovered first, and uncleaved $G_{D3}$ was eluted in later fractions. The incubation of cleaved $G_{D3}$ with proteins resulted in the formation of Schiff bases between the cleaved ganglioside and ε-aminolysyl groups. They were reduced with sodium cyanoborohydride to form stable secondary amine bonds (28). The reaction was monitored by HPTLC, which showed a decreasing ratio of the cleaved $G_{D3}$ to a resorcinol positive band at the origin, indicating the formation of neoglycoconjugates. The reaction was generally completed after incubation for 48 h at 37°C. Disialyllactose was readily remov-

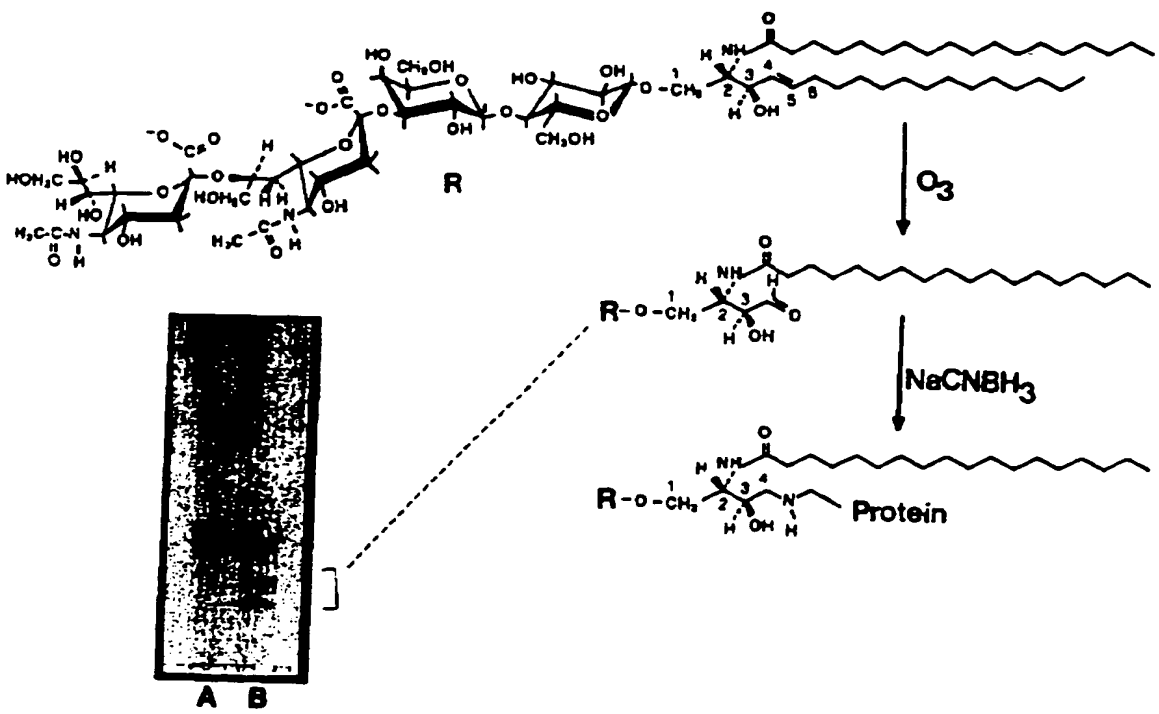


Fig. 1. Synthesis of $G_{D3}$ protein conjugates after ozone cleavage and reductive amination. *Inset*, HPTLC of $G_{D3}$ before (*A*) and after (*B*) ozone cleavage.

able by dialysis, and the excess of cleaved $G_{D3}$ was removed by passage through a detergent-removing column. The degree of coupling was determined by sialic acid and protein determinations. The weight ratio of $G_{D3}$ to proteins in the different conjugates, shown in Table 1, depended on the accessibility of lysine groups in the proteins. The average yield of $G_{D3}$ coupled to proteins was 30%. $G_{D3}$ conjugates prepared in this way were reactive with anti-$G_{D3}$ mAb R24 by Western blot analysis, although the $G_{D3}$-aldehyde derivative itself was not reactive by ITLC (data not shown).

**Oligosaccharide Conjugation.** The carbohydrate part of $G_{D3}$, disialyllactose, was coupled to proteins utilizing two methods. The first method, reductive amination, resulted in conjugation of the open ring form of the glucose to proteins (26). The method required a long incubation of the oligosaccharide with proteins, and the yield was less than 20%. In the second method (27), involving *N*-acroloylation of the terminal glucose, the oligosaccharide was coupled to proteins with a closed ring formation. None of these oligosaccharide conjugates showed reactivity with mAb R24 by Western blot analysis (data not shown).

**Induction of a Serological Response against $G_{D3}$ by Immunization with $G_{D3}$-Protein Conjugates.** All vaccines were well tolerated. Mice were observed for at least 6 months, and neither acute nor systemic toxicity was detected. The serological response to immunization with $G_{D3}$ or $G_{D3}$-protein conjugates, using QS-21 as adjuvant, is shown in Table 1. QS-21 was used because we had previously demonstrated its superiority over other adjuvants with another carbohydrate antigen-KLH conjugate vaccine (29). In ELISA, preimmunization sera showed no IgM or IgG antibodies reactive with $G_{D3}$. Immunization with unconjugated $G_{D3}$ did not induce the production of $G_{D3}$ antibodies. Immunization with $G_{D3}$ conjugates, on the other hand, was effective in inducing antibody production. Of the five proteins used in the preparation of the conjugates, KLH showed the

Table 1 *Antibody response to immunization with different vaccines containing $G_{D3}$ or disialyllactose conjugated to carrier proteins*

| Vaccine + QS-21 | No. of mice | $G_{D3}$-protein weight ratio[a] | Reciprocal ELISA peak titer against $G_{D3}$ | |
|---|---|---|---|---|
| | | | IgG | IgM |
| $G_{D3}$ | 5 | | 0 (5) | 20 (3), 0 (2) |
| $G_{D3}$/KLH[b] | 5 | 0.33 | 0 (5) | 160, 40, 20 (3) |
| $G_{D3}$-KLH[c] | 14 | 0.69 | 10,240 (2), 5,120 (2), 2,560 (3), 1,280 (2), 80 (2), 40 (2), 0 | 2,560, 1,280 (2), 640, 320 (3), 160 (2), 80 (3), 20, 0 |
| $G_{D3}$-cBSA[c] | 15 | 0.77 | 2,560 (2), 320 (2), 160, 80 (2), 40 (4), 20 (2), 0 (2) | 80 (2), 40 (2), 20 (7), 0 (4) |
| $G_{D3}$-OMP[c] | 15 | 0.93 | 2,560, 80 (4), 20 (3), 0 (7) | 1,280, 320 (2), 160 (7), 80 (4), 40 |
| $G_{D3}$-MAP[c] | 10 | 1.0 | 40, 0 (0) | 160 (2), 40 (4), 20 (3), 0 |
| $G_{D3}$-Polylysine | 10 | ND | 0 (10) | 320, 160 (4), 80, 40, 20 (2), 0 |
| Disialyllactose-KLH[d] | 4 | 0.055 | 0 (4) | 160 (3), 80 |
| Disialyllactose-cBSA[d] | 4 | 0.16 | 20, 0 (3) | 40, 20 (3) |
| Disialyllactose-KLH[e] | 4 | 0.25 | 20, 0 (3) | 40 (2), 0 (2) |
| Disialyllactose-cBSA[e] | 4 | 0.34 | 0 (4) | 0 (4) |
| Disialyllactose-Polylysine | 5 | ND | 0 (5) | 80 (3), 40 (2) |

[a] Protein and ganglioside content were determined by BioRad protein assay and by neuraminic acid determination according to the method of Svennerholm (25).
[b] $G_{D3}$ and KLH were mixed prior to immunization.
[c] $G_{D3}$ was covalently attached to proteins prior to immunization after ozonolysis as described in "Materials and Methods."
[d] Disialyllactose was conjugated to KLH and cBSA by reductive amination according to the method of Gray (26).
[e] Disialyllactose was conjugated to KLH, cBSA, and poly-L-lysine after *N*-acroloylation and Michael addition according to the method of Roy and Lafferière (27).

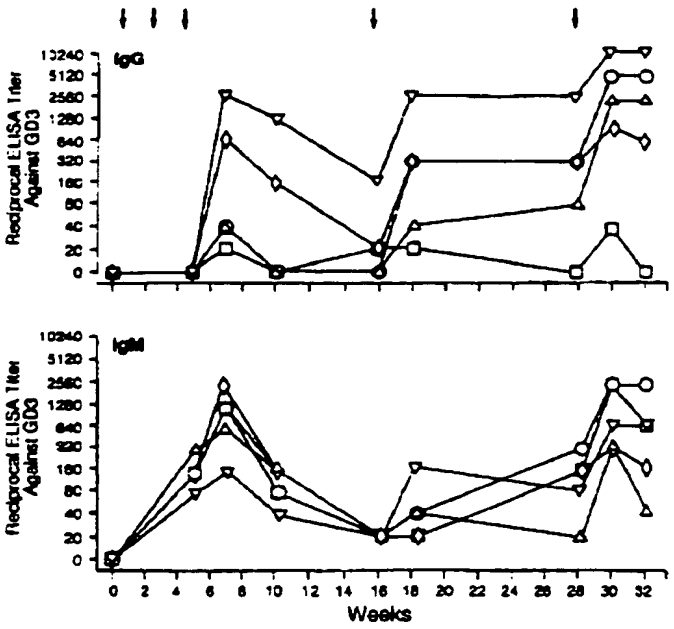


Fig. 2. Time course of $G_{D3}$ antibodies induced in representative mice immunized with $G_{D3}$-KLH and QS-21 vaccine. Each symbol represents an individual mouse. Arrows, time of vaccination.

strongest immunogenicity, resulting in a median titer of 1:320 for IgM and 1:2560 for IgG antibodies. The specific isotype profile was determined with subclass-specific secondary rabbit anti-mouse antibodies. Antigen-specific antibodies were found to be predominantly of the IgG1 subclass. Antigen-specific IgG2a and IgG2b antibodies were found only in traces, and no IgG3 or IgA antibodies were detected.

In contrast to immunization with $G_{D3}$ conjugates, immunization with $G_{D3}$-oligosaccharide conjugates induced only a weak IgM response to $G_{D3}$ and no IgG response.

Sequential IgM and IgG antibody titers against $G_{D3}$ for five mice immunized with $G_{D3}$-KLH and QS-21 are shown in Fig. 2. IgM titers peaked 2 weeks after the third vaccination and declined by the time of the first booster immunization at week 16. The first booster immunization had no significant impact on IgM titers, but the second booster immunization at week 28 increased IgM titers to the peak level seen after the third vaccination of the initial series. IgG titers also rose up to 2 weeks after the third vaccination and decreased by the time of the first booster vaccination but rapidly increased after the booster to previous peak titers. IgG titers remained at this level for 10 weeks, with a further increase after the second booster in most mice. The evidence for a secondary immune response after the booster immunization was therefore equivocal. The response was clearly more rapid than after the initial immunization and lasted longer, but the increase in titer was not comparable to booster responses seen with classical T-cell-dependent antigens.

**Specificity of the Serological Response to Immunization with $G_{D3}$-Protein Conjugates.** The specificity of the serological response to immunization with $G_{D3}$-protein conjugates and QS-21 was analyzed by dot-blot immune staining and ITLC. An example of dot-blot immune stain analysis is shown in Fig. 3. Preimmune sera and immune sera showing high $G_{D3}$-antibody titers in ELISA were tested on nitrocellulose strips that had been spotted with $G_{D3}$ (bbm) or $G_{D3}$ (mel) and purified structurally related gangliosides: $G_{M3}$, $G_{D2}$, $G_{D1b}$, and $G_{T3}$. As expected on the basis of the ELISA results, preimmune sera showed no reactivity. In contrast, sera obtained after immunization with KLH conjugates of $G_{D3}$-ganglioside reacted with $G_{D3}$ (bbm) (the immunogen) or $G_{D3}$ (mel), but not with the other gangliosides except $G_{T3}$ in some cases, a pattern also seen in tests of the mouse monoclonal IgG3 antibody R24, the reagent by which high cell surface expression of $G_{D3}$ on human melanoma cells was first defined (20). The same specificity pattern was seen in dot-blot immune stain tests of sera from mice immunized with other $G_{D3}$-protein conjugates, the only exception being high-titer sera (by ELISA) from mice immunized with $G_{D3}$-cBSA, which showed no reactivity with $G_{D3}$ or the other gangliosides.

ITLC permits specificity analysis of ganglioside antibodies in tests on tissue extracts. Examples of tests with high-titer sera from mice immunized with $G_{D3}$-KLH and QS-21 are shown in Fig. 4. The sera were tested at a dilution of 1:150 on ganglioside extracts of human brain, neuroblastoma, and melanoma, as well as $G_{D3}$ (bbm) that had been used for immunization. The figure shows HPTLC ganglioside patterns of these reagents after staining with resorcinol, as compared with the patterns of reactivity exhibited after exposure to sera from immunized mice or mAb R24. As can be seen in the resorcinol-stained panel, the predominant gangliosides in the brain tissue extract are $G_{M1}$, $G_{D1a}$, $G_{D1b}$, and $G_{T1b}$, whereas the neuroblastoma extract shows $G_{D2}$ and $G_{M2}$ in addition, and the melanoma extract contains mainly

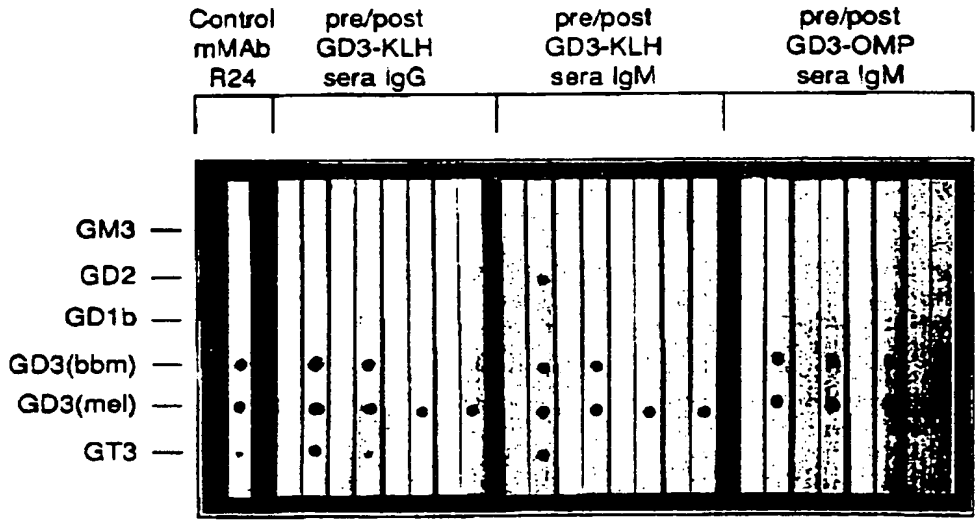


Fig. 3. Dot-blot immune stain assay for IgM and IgG antibodies in sera of mice immunized with $G_{D3}$-KLH and $G_{D3}$-OMP conjugates and QS-21. Antigen standards were applied to nitrocellulose strips in equal amounts (0.5 μg) and were allowed to react with pre/postimmunization serum from individual mice.

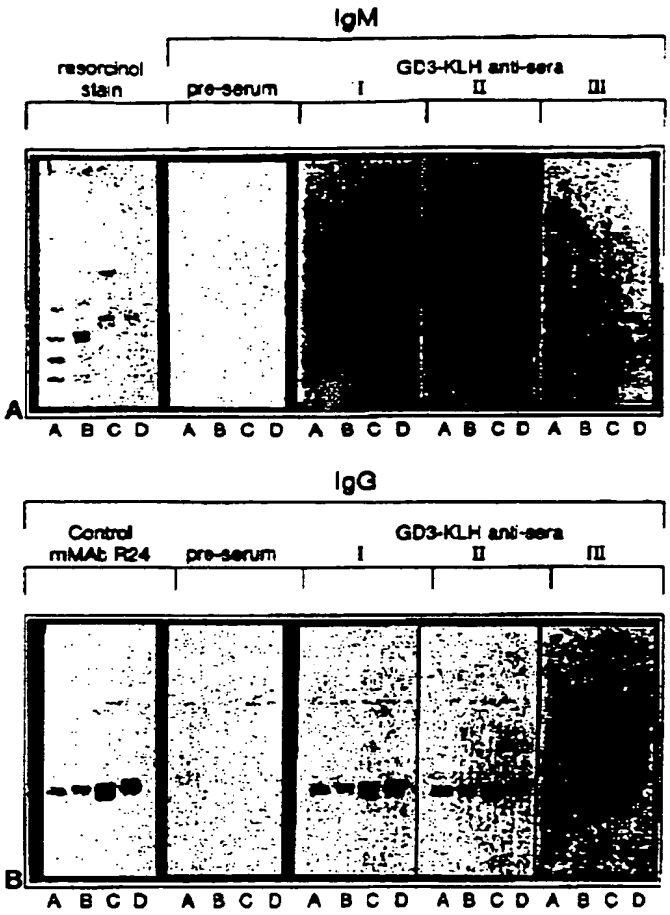


Fig. 4. Immune thin-layer chromatograms of three representative mouse sera after vaccination with $G_{D3}$-KLH conjugate and QS-21. IgG and IgM antibodies in pre- and postvaccination sera and anti-$G_{D3}$ mMab R24 were tested on human brain gangliosides (*A*), neuroblastoma gangliosides (*B*), melanoma gangliosides (*C*), and $G_{D3}$ (*D*) (bbm). Gangliosides were chemically stained with resorcinol-HCl reagent to demonstrate the ganglioside composition of each sample.

$G_{D3}$ and $G_{M3}$. Reactivity of IgG antibodies in postimmunization sera, as well the reactivity of IgG3 mouse monoclonal antibody R24, was restricted to $G_{D3}$ (Fig. 4*b*). The high-titer IgM antibodies, on the other hand, showed weak cross-reactivity with other gangliosides and sulfatide in the brain extract (Fig. 4*a*).

Sera from mice immunized with other $G_{D3}$ conjugates were tested in the same way (at lower dilution) and showed the same specificity with the exception, again, of high-titer sera from mice immunized with $G_{D3}$-cBSA, which showed no ganglioside reactivity (data not shown).

**Cell Surface Reactivity of Immune Sera Determined by FACS Analysis.** Sera from mice were tested for binding to cells of the melanoma cell line SK-MEL-28, a cell line known to express cell surface $G_{D3}$. A representative example of a FACS analysis utilizing a fluorescein isothiocyanate-labeled secondary goat anti-mouse antibody is shown in Fig. 5. Sera before and after immunization with $G_{D3}$-KLH and QS-21 were tested. Preimmunization serum stained 8% of the target cells, postimmunization serum 92%.

## DISCUSSION

Conjugation of poorly immunogenic antigens to highly immunogenic carrier molecules is a well-known approach to augmenting immunogenicity. Ganglioside molecules are so small, however, that linkage to carrier molecules without affecting the relevant antigenic epitopes is difficult. We have shown previously that modifications of $G_{D3}$ in its carbohydrate portion (*i.e.*, conversion of sialic acid carboxyl groups to amides or gangliosidels or lactones) results in markedly increased immunogenicity. However, antibodies produced in response to these $G_{D3}$ derivatives show no cross-reactivity with native $G_{D3}$ (11, 30). Covalent attachment of proteins to the sialic acid molecules of $G_{D3}$ was therefore not attempted in the present study. Our initial approach involved conjugation of $G_{D3}$ oligosaccharide (disialyllactose) via the terminal glucose in open- or closed-ring configuration to KLH or polylysine, but these conjugates were not recognized by the anti-$G_{D3}$ mAb R24 or by mouse antisera to $G_{D3}$, and mice immunized with the conjugates did not produce $G_{D3}$ antibodies. Subsequently, we coupled $G_{D3}$ to proteins via its ceramide portion without alteration of the carbohydrate moiety. The ceramide was cleaved with ozone at the double bond of the sphingosin base, and coupling to proteins was accomplished by reductive amination. Cleavage of gangliosides by ozonolysis and subsequent conjugation with proteins by this method has not been described, and it has been generally assumed that the aldehyde intermediates of gangliosides would be unstable. Fragmentation, initiated by hydroxy ions under alkaline conditions, has been reported. Migration of the double bond would result in $\beta$-elimination, causing release of the oligosaccharide moiety (22, 31). We found, however, that the aldehyde was sufficiently stable at neutral pH to permit Schiff base formation with amino groups of proteins, so that $\beta$-elimination was not a major problem. The overall yield was 30%. These $G_{D3}$ aldehyde-protein conjugates showed reactivity with $G_{D3}$ antibodies by Western blot analysis, indicating that the immunodominant epitopes were intact in these $G_{D3}$ conjugates. However, reactivity of the $G_{D3}$-aldehyde derivative with mAb R24 by ITLC could not be shown. This may be due to its relatively unstable nature, resulting in $\beta$-elimination and release of oligosaccharide during the immune stain incubation period, or simply to the fact that the $G_{D3}$-aldehyde derivative may not adhere to the thin-layer plate sufficiently for serological detection.

Earlier studies describe oxidative ozonolysis of the glycosphingolipid olefinic bond, resulting in a carboxyl group that could be conjugated with carbodiimide to $NH_2$ groups of modified glass beads, agarose gel, or other macromolecules (32, 33). This method, however, is of limited use for the conjugation of gangliosides to carrier proteins because it requires acetylated, methyl ester derivatives of gangliosides to avoid coupling via the sialic acid carboxyl group. Deacetylation after conjugation under basic conditions is necessary, conditions most proteins cannot be exposed to without degradation.

Once the conjugation method was established, several protein carriers were considered, based on previous work by others. Lowell *et al.* (34) described an elegant system that resulted in high-titer antibody responses as a consequence of anchoring bacterial carbohydrate and peptide antigens via a synthetic, hydrophobic foot in OMPs f *Neisseria meningitidis* (35). This system was directly applicable to gangliosides because of their amphipathic nature. In previous studies, we adsorbed gangliosides onto OMP by hydrophobic interaction, and we were able to induce high-titer IgM responses (36). Covalent attachment was utilized in the current study, but $G_{D3}$-OMP conjugates induced only occasional IgG responses, and the IgM response was not increased. Conjugation with cationized BSA, which has been reported to be a potent carrier for protein antigens (37), resulted in high-titer IgG antibodies detected by ELISA, but immune stains indicated that the response was not $G_{D3}$-specific. Another appealing carrier is the MAP system described by J. P. Tam (38, 39). MAPs consist of four or eight dendritic peptide arms, containing B- and T-cell epitopes, attached to an oligomeric branched lysine core. The antibody response to peptides was dramatically increased when these constructs were

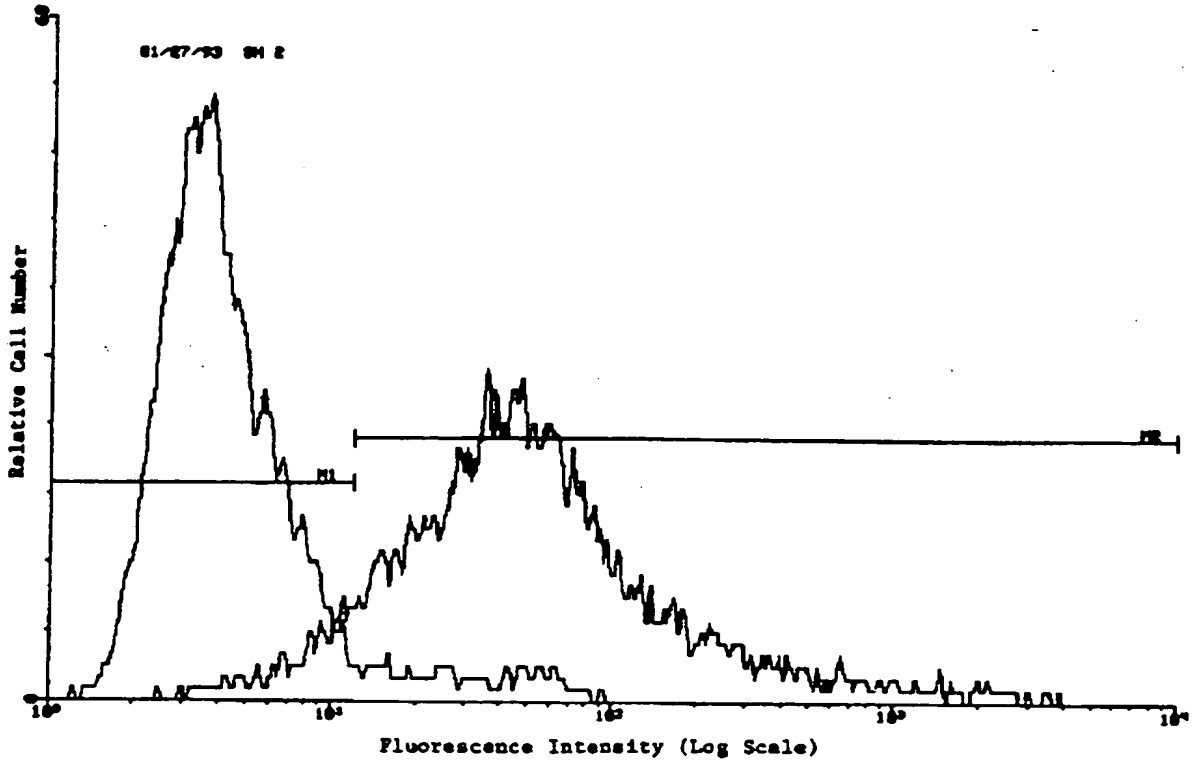


Fig. 5 Representative FACS analysis of mouse serum reactivity prior to (peak at 3) and after (peak at 50) immunization with $G_{D3}$-KLH and QS-21 tested on melanoma cell line SK-MEL-28.

used. When we attached $G_{D3}$ to the amino terminal end of the MAP structure containing a malarial T-cell epitope, only a moderate IgM response against $G_{D3}$ was detected, and there was no detectable IgG response. Conjugation of $G_{D3}$ to polylysine resulted in a medium-titer IgM response and no IgG response, despite the high density of $G_{D3}$ epitopes on these constructs.

The carrier that proved to be most effective in enhancing the antibody response to $G_{D3}$ in this series was KLH. Immunization with $G_{D3}$-KLH consistently induced long-lasting production of IgM and IgG antibodies against $G_{D3}$ at high titers. In comparing KLH with cBSA, OMP, MAP, and polylysine, it is difficult to know exactly why KLH is a superior carrier for $G_{D3}$. The sheer size and antigenic complexity of KLH stand out as a possible aid to antigen processing and recruitment of T-cell help across a broad range of T-cell specificities. The very qualities that make KLH cumbersome to work with are probably responsible for its unique effectiveness as a carrier in conjugate vaccines. KLH has not been widely used as a carrier for conjugate vaccines in humans because its size and heterogeneity make vaccine construction and standardization difficult.

Our hope was that conjugate vaccines would convert the T-cell-independent response against unconjugated $G_{D3}$ seen in our previous studies to a T-cell-dependent response producing high-titer, long-lived, IgG antibodies. This expectation was fulfilled to some extent but not completely. The peak of the IgM response occurred after the third biweekly vaccination as in our previous studies with unconjugated $G_{D3}$, but the antibody titers were significantly higher. The response declined rapidly (as observed before), and additional vaccinations increased IgM titers to previous peak levels. The repeated increase in the titer of IgM antibodies to $G_{D3}$ after booster immunizations differs from the expected response to T-cell-dependent antigens such as proteins, which generally induce little or no IgM response after booster immunizations. For the first time, however, we were able to induce a high-titer IgG response against $G_{D3}$ ganglioside consistently. This response lasted significantly longer than the IgM response and was increased by additional vaccinations, although the response following booster vaccinations was not comparable to the exponential increase often seen with protein antigens. The fact that the $G_{D3}$ antibodies were of the IgG1 subclass indicates that a T-cell-dependent pathway was activated by the $G_{D3}$-KLH conjugate vaccine. The lack of a classical booster effect, however, may reflect the carbohydrate nature of $G_{D3}$ and its status as an auto-antigen. This suggests that T-cell recruitment by ganglioside conjugate vaccines is limited by the nature of the antigen itself. Nevertheless, the high-titer IgM response and long-lived IgG response to vaccination with $G_{D3}$-KLH and QS-21 seen in these experiments represents a striking improvement over the response to unconjugated ganglioside vaccines and can now form the basis for clinical trials of ganglioside-KLH conjugate vaccines in patients with cancers that show increased ganglioside expression.

## ACKNOWLEDGMENTS

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